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Sonic Hedgehog induces proliferation of committed skeletal muscle cells in the chick limb

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SUMMARY

Myogenic Regulatory Factors (MRFs) are a family of transcription factors whose expression in a cell reflects the commitment of this cell to a myogenic fate before any cytological sign of muscle differentiation is detectable. Myogenic cells in limb skeletal muscles originate from the lateral half of the somites. Cells that migrate away from the lateral part of the somites to the limb bud do not initially express any member of the MRF family. Expression of MRFs in the muscle precursor cells starts after the migration process is completed. The extracellular signals involved in activating the myogenic programme in muscle precursor cells in the limb in vivo are not known. We wished to investigate whether Sonic Hedgehog (SHH) expressed in the posterior part of the limb bud could be involved in differentiation of the muscle precursor cells in the limb. We found that retrovirally overexpressed SHH in the limb bud induced the extension of the expression domain of the *Pax-3* gene, then that of the *MyoD* gene and finally that of the myosin protein. This led to an hypertrophy of the muscles in vivo. Addition of SHH to primary cultures of myoblasts resulted in an increase in the proportion of myoblasts that incorporate bromodeoxyuridine, resulting in an increase of myotube number. These data show that SHH is able to activate myogenesis in vivo and in vitro in already committed myoblasts and suggest that the stimulation of the myogenic programme by SHH involves activation of cell proliferation.

Key words: *Sonic hedgehog* (*Shh*), Chick, Limb bud, Virus, Muscle differentiation, Cell signalling

INTRODUCTION

All the myogenic cells forming the striated skeletal muscles of the vertebrate body originate from the somites. Cell lineage experiments have shown that the ventral parts of the somites, forming the sclerotome, give rise to the axial skeleton and ribs and that their dorsal parts or dermomyotomes differentiate into skeletal muscles and dermis (for a review, see Christ and Ordahl, 1995). The back and intercostal muscles constituting the epaxial musculature originate exclusively from the medial halves of the dermomyotomes, while cells derived from lateral dermomyotome migrate lateroventrally to produce the muscles of the ventral body wall and of the limb, forming the hypaxial musculature (Ordhal and Le Douarin, 1992). Determination of the different somitic components takes place under the influence of environmental signals (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992). The nature and source of these environmental influences is the subject of intensive investigations. It is now accepted that axial structures (neural tube and notochord) stimulate the process leading to differentiation of epaxial skeletal muscles. However, in vitro studies have yielded contradictory results concerning the precise source of these signal(s) (Buffinger and Stockdale, 1994, 1995; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995; Ponwall et al., 1996). These controversial data might reflect the existence of a combination of signals initiating, activating or maintaining myogenesis.

Axial structures do not appear to be required for the differentiation of hypaxial muscles. Ablation of the neural tube and the notochord leads to a striking absence of vertebral axial muscles, while the differentiation of hypaxial musculature of limb and body wall is not imparied (Teillet and Le Douarin, 1983; Rong et al., 1992). In chick embryos subjected to neuralectomy and notochordectomy, cells of the medial somitic half die within 24 hours following the excision, whereas myoblasts derived from the lateral half of the somite survive, migrate and differentiate, suggesting the existence of a distinct environmental inductive influence on the differentiation of hypaxial skeletal muscles (Teillet and Le Douarin, 1983; Rong et al., 1992). Lateral-somitic lineage specification results from signals emanating from lateral plate mesoderm (Pourquié et al., 1995, 1996; Cossu et al., 1996) and dorsal ectoderm (Kenny-Mobbs and Thorogod, 1987; Fan and Tesier-Lavigne, 1994; Cossu et al., 1996). The current

view is that the lateral plate produces a diffusible signal, problably BMP-4, which prevents muscle terminal differentiation in the lateral part of the somite (Pourquié et al., 1996; Tonegawa et al., 1997). This maintenance in an undifferentiated state of already committed muscle cells would allow their migration to the limbs at the brachial and lumbar levels, and to the body wall at the thoracic level. In consequence, differentiation of peripheral muscle precursors originating from the lateral parts of somites is delayed by 2 days compared to differentiation of axial muscles (Buckingham, 1992). This delay is accompanied by the maintenance of the expression of the transcription factor *Pax-3*, belonging to the family of paired box genes, whose transcripts are expressed in migrating myoblasts (Goulding et al., 1994; Williams and Ordahl, 1994). *Pax-3* expression is thought to be a marker of an undifferentiated state of the precursor cells giving rise to skeletal muscle (Epstein et al., 1995; Maroto et al., 1997; Tajbakhsh et al., 1997). Thus, Pax-3-positive cells will express members of the MRF family only when they have reached their destination (Sasoon et al., 1989; Williams and Ordahl, 1994; Tajbakhsh and Buckingham, 1994). Expression of *MyoD* and *Myf-5* genes takes place in proliferating myoblasts prior to any overt terminal differentiation into myotubes characterised by the appearence of structural muscle proteins (Williams and Ordahl, 1994).

The extracellular signals involved in activating the myogenic programme in the muscle precursor cells that reach the limb in vivo are not known. How myogenic differentiation is initiated after the migration process and regulated in the limb is an important question. *Sonic hedgehog* (*Shh*), encodes a secreted protein which has been shown to be involved in the patterning of the vertebrate nervous system (Roelink et al., 1994), paraxial mesoderm (Jonhson et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995) and limb (Riddle et al., 1993). During limb development, pattern along the anteroposterior axis is specified by a group of cells located in the posterior mesenchyme of the limb bud, termed ‘zone of polarising activity’ (ZPA) (Saunders and Gasseling, 1968). *Shh* transcripts are expressed in the posterior part of the mesenchyme of emerging wing buds from stage 17 (3 days) in a region that corresponds to the ZPA (Riddle et al., 1993; Laufer et al., 1994). SHH-expressing cells grafted to the anterior part of the limb have the property of inducing digit duplication (Riddle et al., 1993). Moreover, the transmembrane protein Patched (PTC), considered to be the (one of the) receptor(s) for the SHH signal (Marigo et al., 1996a; Stone et al., 1996) is also expressed in the posterior mesenchyme of the limb bud, but in a domain broader than that of *Shh* (Marigo et al., 1996b; Goodrich al., 1996). Expression of the SHH/PTC complex (Laufer et al., 1994; Marigo et al., 1996b) begins after the myoblast precursors have migrated to the limb bud (Chevalier et al., 1977; Christ et al., 1977; Chevalier, 1978; Seed and Hauschka, 1984; Kenny-Mobs and Thorogood, 1987) but before activation of the MRFs (Williams and Ordahl, 1994; Tajbakhsh and Buckingham, 1994) and subsequent terminal myogenic differentiation have taken place. This suggests that the incoming muscle precursors could be a target for SHH signalling when they have reached their destination.

In order to understand the role of SHH signalling in limb muscle development, we retrovirally overexpressed *Shh* in the early limb bud in vivo and looked for subsequent changes in muscle markers and muscle morphology. We found that ectopic expression of SHH in the limb bud sequentially induced the extension of the expression domains of the *Pax-3* and *MyoD* genes and of the myosin protein. These events led to an hypertrophy of limb muscles. We also showed that SHH increases the proliferation of myoblasts in vitro which results in an increase of myotube number, suggesting that the enhancement of peripheral muscle differentiation by SHH involves proliferation of already committed myoblasts.

MATERIALS AND METHODS

Chick embryos

Fertilised White Leghorn chick eggs (HAAS, Strasbourg, France) were incubated at 37°C and embryos staged according to Hamburger and Hamilton (HH;1951). All grafting experiments were performed in ovo.

Production of SHH-expressing cells

Production of Shh/RCAS-expressing cells

Infectious Shh/RCAS viruses were produced in Chick Embryo Fibroblasts (CEF) as described by Duprez et al. (1996a). Briefly, CEF were isolated from 10 day-old 0 line embryos (BBSRC; Institute for Animal Health, Compton, Berkshire, UK) and grown in DMEM (Gibco, BRL) containing 8% (v/v) foetal calf serum and 2% (v/v) chick serum supplemented with antibiotics. CEF were transfected transiently with retroviral recombinant DNA using Transfectam (Gibco, BRL) according to the manufacturer’s instructions.

Production of SHH-expressing QT6 cells

The chicken SHH coding region was inserted in the pBK plasmid (Stratagen) by Hermann Rohrer (Germany). A stable QT6 cell line producing SHH/pBK was established as described by Duprez et al. (1996b). Stable transfectants were selected in G418 and the best clone was chosen according to its ability to induce digit duplication in vivo.

Grafting of retrovirus-infected cells

Retrovirus-expressing cells were prepared for grafting as described by Duprez et al. (1996a,b). Pellets of approximately 100 μm in diameter were grafted into the limbs of White Leghorn embryos at various stages of development (Stage HH18-23). Embryos were harvested and processed for in situ hybridisation of whole mounts or tissue sections. In some cases, embryos were stained with alcian blue and cleared in methyl salicylate to determine digit pattern.

Chick myoblast primary cultures

Primary muscle cell cultures were prepared from ventral and dorsal muscle masses of the forelimb or pectoral muscles removed from 7-day-old chick embryos. Cells were seeded after mechanical dissociation in 35 mm plastic dishes (Costar) at a density of 2×10^5/ml in Dulbecco’s Minimum Eagle Medium (DMEM) containing 10% (v/v) fetal calf serum and 1% (v/v) chick serum and cultured overnight. The next day, myoblast pimary cultures were grown in the same medium or with the supernatants from 0-line cells transiently transfected with Shh/RCAS or QT6 cells stably transfected with SHH/pBK. After 24, 48 or 72 hours myoblast cultures were fixed in 4% (w/v) paraformaldehyde for 1 hour at 4°C. In situ hybridisation and immunohistochemistry were perfomed as described below.

In situ hybridisation to whole mounts and tissue sections

Embryos were fixed in 4% (v/v) formaldehyde and processed as

previously described for in situ hybridisation to whole mounts (Pourquié et al., 1996). In situ hybridisation of digoxigenin-labelled RNA probes to tissue cryostat sections was performed as described by Strähle et al. (1994). In situ hybridisation of ^{35}S-labelled RNA probes to tissue wax sections was performed as described by Duprez et al. (1996a).

Probes for chicken *Shh* and *Ptc* transcripts were prepared as described by Riddle et al. (1993) and Marigo et al. (1996b), respectively. Probes for *Pax3* and *MyoD* transcripts were prepared as described by Pourquié et al. (1996). Probe for chicken *Fgf-4* was prepared as described by Niswander et al. (1994).

Immunohistochemistry

Muscle cells were detected on sections and in cultures using the monoclonal antibody against sarcomeric myosin heavy chain, MF20 (Developmental Hybridoma Bank, University of Iowa, Iowa City) and using a polyclonal antibody against desmin (Sigma, diluted 1/100, v/v). The bromodeoxyuridine (BrdU) was detected using a monoclonal antibody against BrdU (Amersham, Life Sciences, diluted 1/100, v/v).

Myoblast proliferation analysis

Myoblasts were cultured as described above. They were stopped 24, 48 and 72 hours after the platting. Immunostaining using an anti-desmin antibody was performed. An hemalun/hematoxilin stain was used in order to visualise the muscle cell nuclei. For each time point, 20 randomly selected fields of 0.135 mm^2 from four or five different experiments were chosen, and the nuclei of desmin-positive cells were counted. The average number of the desmin-positive cells of the control cultures and the SHH-treated cultures were plotted against the time in culture.

To label cells during the S-phase, BrdU (Amersham, Life Sciences) was added to the culture medium at a final concentration of 10 μM for the final 8 hours of the culture. After 24 hours, the cultures were fixed in 4% (w/v) paraformaldehyde for 1 hour at 4°C. Double immunohistochemistry was performed using, the monoclonal antibody against BrdU followed by the polyclonal antibody against desmin. Forty randomly selected fields of 0.135 mm^2 from two different experiments were chosen. In each field the nuclei of desmin- and BrdU-positive cells, and the total number of desmin-positive cells were counted, in order to find the proportion of desmin-positive cells that are BrdU positive. The average number of the double-positive cells out of the total number of desmin-positve cells was plotted, for SHH-treated cultures and for control cultures, against the time in culture.

Muscle fibers counts

Tranverse sections of middle-grafted wings at E10, stained with the MF20 antibody, were used to count muscle fibre number. 100 μm^2 areas were chosen in 20 sections. The muscle fiber counts in Shh/RCAS-infected and control wings were performed on sections cut from the same parts of each wing.

RESULTS

Overlapping of the *Ptc*, *Pax3* and *MyoD* expression domains in the developing forelimb

The transcripts coding for the SHH protein are localised in a restricted posterior region of the limb corresponding to the ZPA (Riddle et al., 1993). The SHH protein could never be detected outside its mRNA expression domain (Lopez-Martinez et al., 1995; Marti et al., 1995, Yang et al., 1997). However, the *Ptc* receptor has a broader expression domain than *Shh* suggesting that SHH might act beyond the area where it is synthesized, thus providing one measure of the distance that SHH could diffuse (Marigo et al., 1996b; Marigo and Tabin, 1996, Goodrich et al., 1996). In order to determine the relationship between the expression of the SHH/PTC complex and limb muscle development, we compared the expression of *Ptc* with that of specific genes expressed in muscle lineage. *Pax3* was used as a marker of the migrating and undifferentiated myoblasts (Williams and Ordahl, 1994). *MyoD* was chosen as a marker showing the commitment to a myogenic fate (Ponwall and Emerson, 1992; Williams and Ordahl, 1994). Serial sagittal sections of the wing (Fig. 1A) were prepared at different stages after completion of the migration of the muscle progenitors. At stage HH20 (E3.5), myogenic precursor cells visualised by *Pax-3* expression are present throughout the limb and are included within the *Ptc* expression domain (data not shown). At stage HH23, *Ptc* transcripts are present in both anterior and posterior regions of the limb bud in a graded expression pattern from the posterior (strongest expression) to the anterior part (Fig. 1B). The myogenic precursor cells of the ventral and dorsal muscle masses expressing *Pax3* mRNA (Fig. 1D,E) are still included within the *Ptc* expression domain (Fig. 1B,C). *MyoD* mRNAs

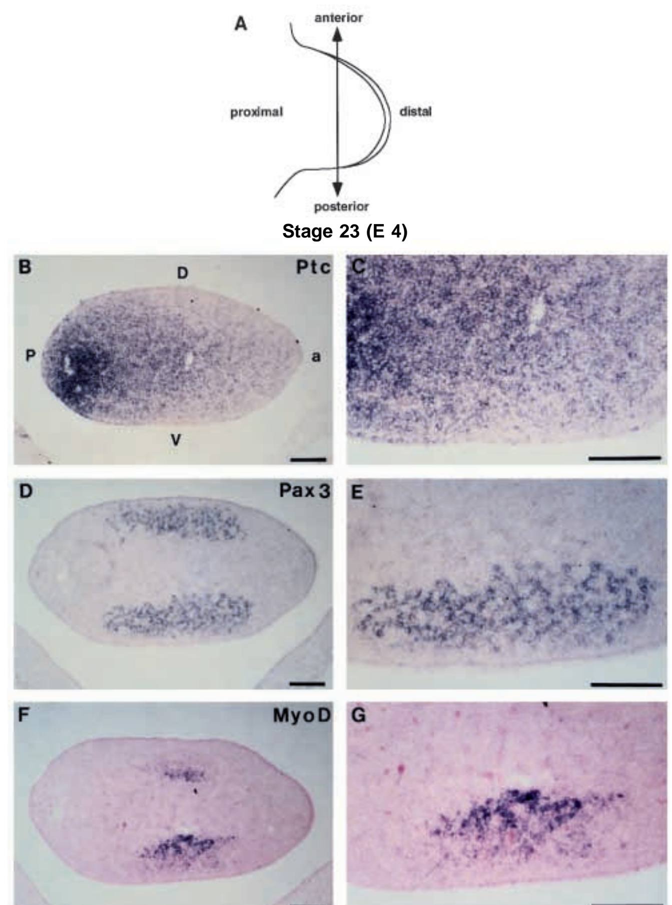

Fig. 1. Comparison of *Ptc* and muscle marker expression domains in stage HH23 embryonic chick wing. Adjacent sagittal sections (A) were hybridised with DIG-labelled antisense probes for *Ptc* (B,C), *Pax-3* (D,E) and *MyoD* (F,G). C,E and G show the ventral areas from B,D and F, respectively, at twice the magnification. a, anterior; p, posterior; D, dorsal and V, ventral. Scale bars, 120 μm.

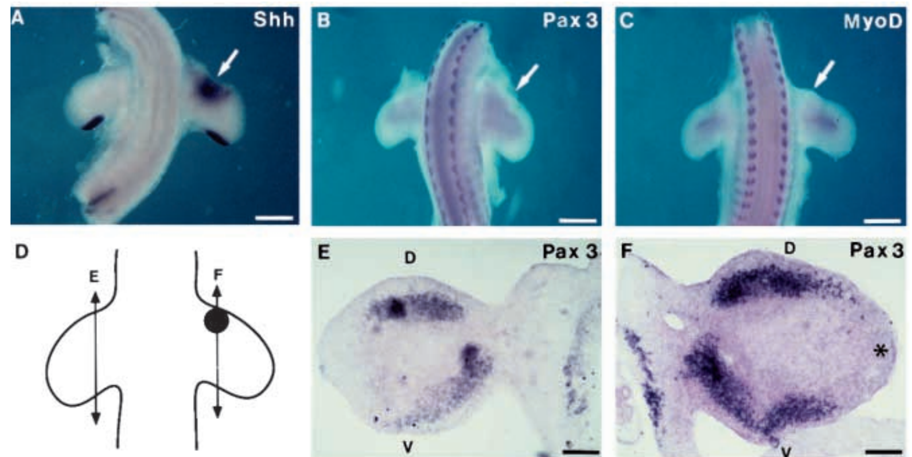

Fig. 2. Ectopic SHH is able to induce extension of *Pax-3* expression domain before any overt expression of *MyoD*. (A) 24 hours after grafting Shh/RCAS-expressing cells, viral transcripts were detected in whole-mount preparations by in situ hybridisation with a probe against *Shh*. Ectopic *Shh* is present (arrow) in anterior region of the wing. Distribution of *Pax-3* (B) and *MyoD* (C) transcripts in whole-mount preparations 24 hours after grafting (stage HH23). Arrows show the positions of the grafted cells. Sagittal sections (D) were prepared from manipulated embryos fixed at stage HH22, from contralateral left wing (E) and the Shh/RCAS-grafted right wing (F) and hybridised with Pax-3 probe. * shows the position of the grafted cells. D, dorsal; V, ventral; Scale bars A,-C, 750 µm; E,F, 150 µm.

were first detected in the dorsal and ventral areas containing the future muscle masses at this stage (Fig. 1F,G). The appearance of *MyoD* transcripts revealed by digoxigenin in situ hybridisation precedes those detected with the ^{35}S in situ hybridisation technique (Williams and Ordahl, 1994), this early detection is however consistent with the results of a reverse transcriptase polymerase chain reaction (RT-PCR) assay carried out by Lin-Jones and Hauschka (1996). The overlapping of the *Ptc*, *Pax-3* and *MyoD* mRNA expression domains suggests that the muscle masses in the limb could be a target tissue for SHH/PTC signalling.

Overexpression of SHH in limb bud mesenchyme enhances the expression of muscle markers

Aggregates of Shh/RCAS-transfected cells (see Methods) were grafted to the anterior margin of stage HH18/20 wing buds. Twenty-four hours later, in situ hybridisation to *Shh* transcripts in whole-mount preparations showed that the virus remains localised to the region of grafted cells (*n*=4; Fig. 2A, arrow). The *Pax-3* expression domain appears larger in the grafted than in the contralateral wing (*n*=6; Fig. 2B), while no striking change was observed in the *MyoD* expression domain (*n*=6; Fig. 2C). It should be noted that at this stage (stage HH24) the MyoD expression has just started (stage HH22/23). In order to investigate whether ectopic SHH has an effect on *Pax-3*-positive cells before any overt *MyoD* expression can be observed, we performed digoxigenin in situ hybridisation on sagittal sections (Fig. 2D) from limbs, 24 hours after grafting. The embryos were grafted at stage HH18 (E3) and fixed at stage HH22 (E4) just before the appearence of *MyoD* expression. The Shh/RCAS-grafted right wing appears bigger and the *Pax-3* expression domain is enlarged (Fig. 2F) when compared to the contralateral left wing (Fig. 2E). Thus, ectopic SHH induces an extension of the *Pax-3* expression domain prior to any overt *MyoD* expression in the forelimb bud.

If the observation is done 48 and 72 hours after grafting, most of the premuscular masses of the limb were found to be infected with the Shh/RCAS virus (*n*=7; Fig. 3A). As soon as 48 hours after grafting, the shape of the grafted bud had changed: it took on the classic shape of a duplicated wing, broader distally (Fig. 3B; arrowhead), while also being thicker proximally (Fig. 3B; arrow). Analysis of *MyoD* expression by whole-mount in situ hybridisation showed that the proximal enlargement of the manipulated right wing corresponds to an extension of the *MyoD* expression domain compared to the control left wing (*n*=5; Fig. 3B). Three days after anterior grafts, embryos display a proximal *MyoD* expression domain more extended (*n*=3; Fig. 3D, arrow) than in the contralateral wing (Fig. 3C). This extended domain is correlated with the proximal thickness (Fig. 3D, arrow).

Seventy-two hours after Shh/RCAS-expressing cells were grafted into the anterior region of the limb bud, the operated embryos were cut transversely through the forelimb region and hybridised with the Shh probe, revealing the extent of the virus spread (Fig. 4A). Adjacent sections hybridised with Pax-3 (Fig. 4B) and MyoD (Fig. 4C) probes showed that at this stage the brachial muscles are normally individualised in the contralateral left wing. It should be noted that *Pax-3* is still

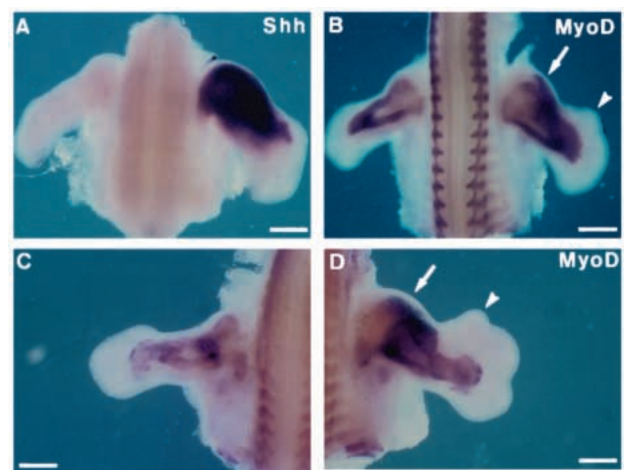

Fig. 3. Ectopic SHH is able to induce extension of *MyoD* expression domain. (A) Viral transcripts were detected in whole-mount preparations by in situ hybridisation with a probe against Shh, 72 hours after anterior grafts at stage 19/20 of Shh/RCAS-expressing cells. Distribution of *MyoD* transcripts in whole-mount preparations 48 hours (B) and 72 hours (C,D) after similar grafts. (C) Control limb; (D) affected limb. Arrowheads show the extension of the distal part of the buds caused by the digit duplication. Arrows show the proximal enlargements of the grafted wings which are MyoD positive. Scale bars, 750 µm.

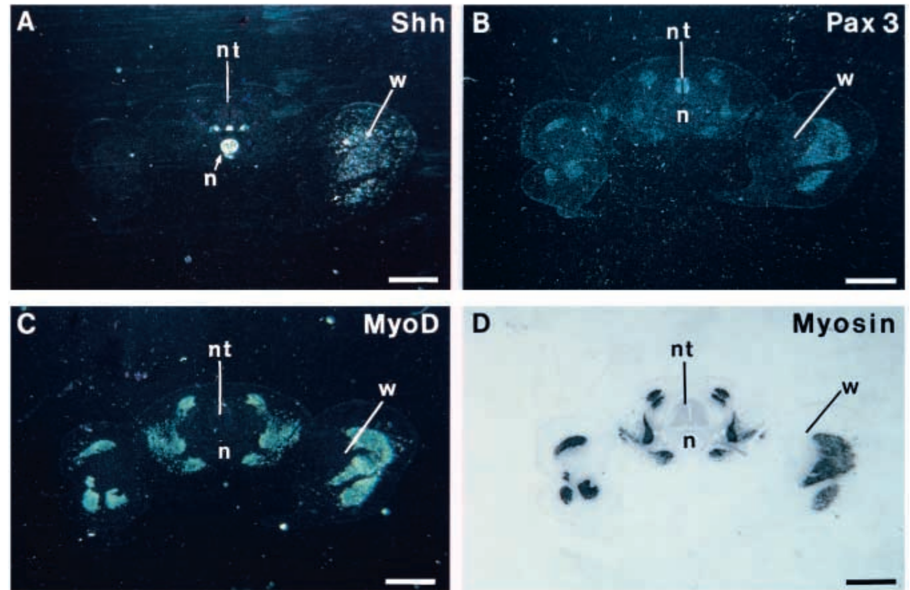

Fig. 4. Expression of muscle genes 72 hours after anterior grafts of Shh/RCAS-expressing cells to right wing buds at stage 19/20. (A) Transverse sections through the forelimb hybridised with a ^{35}S-labelled antisense RNA probe specific for chicken Shh photographed under dark-field illumination showing the extent of the virus spread. Consecutive sections were hybridised with a ^{35}S-labelled antisense RNA probes specific for chicken *Pax-3* (B) and *MyoD* (C), photographed under dark-field illumination and (D) incubated with the MF20 antibody recognising myosin, photographed under bright-field illumination. nt, tube neural; n, notochord; w, wing;. Scale bars, 750 μm.

present at E6 in muscles, but its expression is weak (Fig. 4B). The Shh/RCAS-infected right wing shows an enlargement of the Pax-3-positive (Fig. 4B) and MyoD-positive (Fig. 4C) domains. The muscles do not appear to be individualised in separated masses like the normal wing (Fig. 4B,C). In order to test whether these enlarged muscles had differentiated, we looked for the expression of myosin protein using the MF20 antibody. This protein is a terminal muscle marker specifically expressed in postmitotic muscle cells. Analysis of adjacent sections in experimental embryos shows that the Pax-3- and MyoD-positive enlarged muscles did indeed express the myosin protein (Fig. 3D), demonstrating that these enlarged muscles had differentiated.

Overexpression of SHH in limb mesenchyme induces hypertrophy of the limb musculature in vivo

We then examined the phenotype of the Shh/RCAS-infected limbs at 10 days. Two types of grafts were performed. (1) Shh/RCAS-expressing cells were grafted in the anterior mesenchyme region around stage HH19/20 as before (Fig. 5A). These grafts resulted in the dramatic enlargement of the right wing at ten days (6 days after grafting) (*n*=20; Fig. 5C) in comparison to the non-manipulated wing of the same embryo (Fig. 5B). Analysis of the skeletal pattern showed that the manipulated embryos systematically present extra digits (10 out of 10, data not shown), consistent with the proposed function of SHH as the active molecule of the ZPA (Riddle et al., 1993). (2) In order to avoid the effect of SHH on digit pattern, we grafted Shh/RCAS-expressing cells in the middle of the mesenchyme at stage 21/22 (Fig. 5D). At this stage, digit pattern is already specified and ZPA grafts or application of retinoic acid (RA) or SHH to the bud do not induce any change of digit pattern (Tickle et al., 1975, 1982; Riddle et al., 1993). These middle-grafted wings exhibited no sign of digit respecification (10 out of 10; data not shown). In such manipulated embryos, the grafted wings were thicker (*n*=10; Fig 5F) than the contralateral wings (Fig. 5E). The middle-grafted limbs appear enlarged (Fig. 5F) but to a lesser extent than the anterior-grafted limbs (Fig. 5C), an effect that may be correlated with the extent of the virus spread or with the stage of the embryo at the grafting time.

To determine if the enlargement of the Shh/RCAS-infected limbs corresponds to muscle hypertrophy, we looked for the expression of muscle markers in the middle-grafted limbs at E10. *Shh* RNA revealed by ^{35}S in situ hybridisation, is overexpressed in the manipulated limb (Fig. 6A), though *Shh* is not expressed in the normal limb at this stage (Fig. 6B). In the normal contralateral wing, *MyoD* transcript localisation

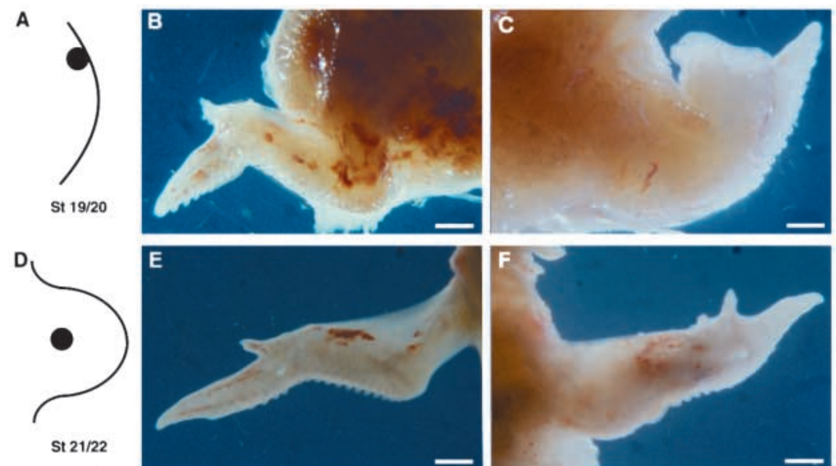

Fig. 5. Effects on limb morphology of grafting Shh/RCAS-expressing-cells. When grafts were placed anteriorly at stage 19/20 (A), 7 days later, at 10 days, the wing is dramatically swollen (C) in comparison to the unaffected contralateral left wing (B). When grafts were placed in the middle of the wing at stage 21/22 (D), in order to avoid the SHH effect on digit patterning, at 10 days, the wing was enlarged (F) in comparison to the unaffected contralateral left wing (E). Scale bars, 1.5 mm.

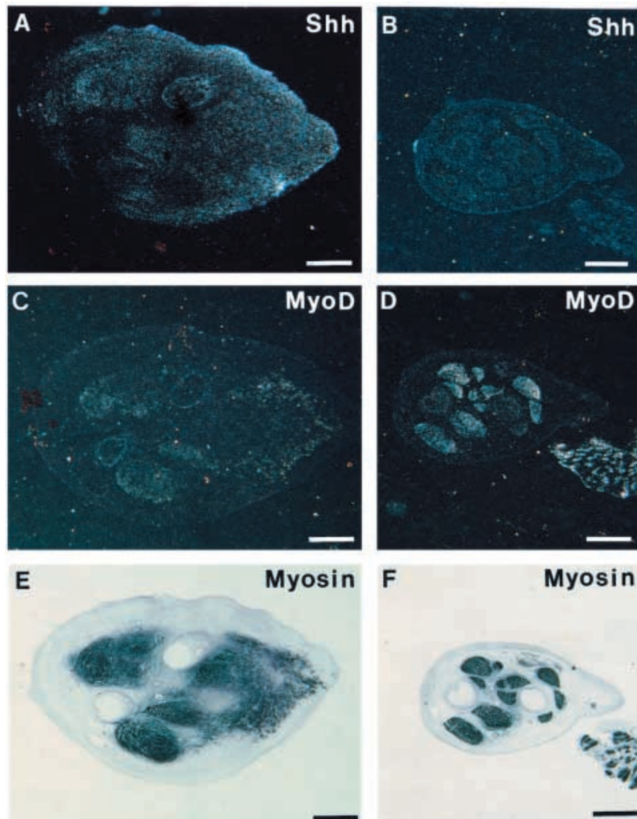

Fig. 6. Analysis of muscle marker expression in middle-grafted wings at 10 days. Adjacent transverse sections of the infected wing (A,C,E) and the contralateral left wing of the same chick embryo (B,D,F) were hybridised with ^{35}S-labelled antisense RNA probes specific for chicken *Shh* (A,B), *MyoD* (C,D) photographed under dark-field illumination or incubated with the MF20 antibody recognising myosin (E,F), photographed under bright-field illumination. The muscles in the anterior region (where ectopic SHH is present) are not organised as individual muscles, but are partially fused. Two muscles appear be clearly separated (E) in the ventroposterior region that seems devoid of ectopic *shh* (A). These muscles look bigger nevertheless (E). a, anterior; p, posterior; D, dorsal; V, ventral. Scale bars, 480 μm.

(Fig. 6D) and myosin protein expression (Fig. 6F) reveal the organisation of muscles in the forearm (Robson et al., 1994). In contrast to the normal wing, in the Shh/RCAS-infected wing *MyoD* expression is not visible (Fig. 6C) in the enlarged and disorganized muscles which all express myosin (Fig. 6F). This suggests that these Shh/RCAS infected-muscles have undergone an accelerated process of differentiation.

In embryos that received grafts of Shh/RCAS-expressing cells in the anterior region of the limb bud, the virus had reached the body wall at 10 days. This induces pectoralis muscle hypertrophy (data not shown), showing that SHH is able to affect all muscle derivatives of the hypaxial muscle lineage.

To determine whether the increase in skeletal muscle masses resulted from hyperplasia or from hypertrophy, we compared the numbers of muscle fibres per unit area in Shh/RCAS-infected right wing in comparison to the control left wing. This showed that muscle fibre density per unit area was roughly similar in control and experimental wings (717±36; in the Shh-infected right versus 570±13; contralateral wing). As the total area of the muscle masses is greater in the affected limbs (Figs 5F, 6A,C,E) than in the contralateral (Figs 5E, 6B,D,F), we conclude that the increase in the muscle masses, induced by SHH, is due to an hypertrophy of muscle.

SHH promotes myogenesis in primary muscle cell cultures

In order to analyse the nature of the SHH effect on limb muscle development, we investigated whether SHH could have a similar effect on primary muscle cell cultures. Primary cultures of muscle cells were prepared from E7 chick embryos. These cultures were treated with supernatant from Shh/RCAS-expressing cells, for 1-3 days. These cells were first hybridised with the Shh probe, showing that most (if not all) muscle cells express *Shh* after 2 days of exposure to Shh/RCAS (Fig. 7A). These cultures exhibited an increase in the number of myotubes visualised with MF20 antibody labelling (Fig. 7B) compared to cultures treated with supernatant from control cells (Fig. 7E) or to untreated cultures (Fig. 7D). Immunocytochemistry using an antibody against the desmin protein gave equivalent results (data not shown). In order to test whether exogenous SHH could have a similar effect, we perfomed experiments using supernatant of QT6 cells stably transfected with the SHH/pBK construct. These cells express SHH protein constitutively. SHH/pBK-treated myoblast cultures also showed an increase in the number of myotubes visualised with MF20 labelling (Fig. 7C), compared to the culture treated with supernatant from non-transfected QT6 cells (Fig. 7F). The effect was nevertheless less marked than with Shh/RCAS-treated cultures (compare Fig. 7B and 7C). SHH does not induce *Shh* in such myoblast cultures (data not shown). These results show that SHH does not need to be expressed in the myoblasts to promote myogenesis, and exogenous SHH is also able to enhance muscle differentiation.

SHH activates myoblast proliferation in vitro

The muscle hypertrophy of the retrovirally Shh-infected limbs in vivo and the myogenesis-promoting effect of SHH in vitro suggested that SHH overexpression results in increasing myoblast proliferation. In order to test this hypothesis, we counted muscle cells (desmin-positive cells) in Shh/RCAS-treated and control cultures. The numbers of cells were plotted against time of culture. There is about a two-fold increase of the cell number in the Shh/RCAS-treated cultures compared to control cultures after 48 and 72 hours of culture (Fig. 8A) reflecting an increase in the proliferation rate in the Shh/RCAS-treated culture in comparison to the control culture. Neither fibroblasts transiently transfected with Shh/RCAS nor the SHH/pBK-QT6-expressing cell line proliferate more quickly than the non-transfected cells and the QT6 cell line, respectively (data not shown).

To confirm the SHH effect on myoblast proliferation, we tested the effect of SHH on the incorporation of bromodeoxyuridine (BrdU) into DNA, after 24 hours of culture. After 24 hours in culture, the total number of desmin-positive cells was slightly increased in SHH-treated culture, as expected (Fig. 8A). There is about a three-fold increase of the BrdU incorporation in desmin-positive cells in SHH-treated cultures

compared to control cultures (Figs 8B, 9). Although this result does not rule out the possibility that SHH promotes cell survival, it does demonstrate that it acts as a mitogen for myoblasts. These results show that SHH enhances proliferation of myoblasts, which in turn leads to an increase of the number of myotubes.

SHH has been shown to induce *Fgf-4* expression in vivo (Niswander et al., 1994; Laufer et al., 1994). FGFs are known to stimulate proliferation of undifferentiated myoblasts (Olson, 1992). We therefore investigated whether SHH could act on myoblast proliferation indirectly, by increasing the level of FGF-4 expression. SHH does not induce *fgf-4* expression in those cultures detected by in situ hybridisation (data not shown). This shows that the SHH effect on muscle cells is not mediated by FGF-4.

DISCUSSION

We show in this work that overexpression of SHH in the limb bud of the E3 chick embryo results in the extension of *Pax-3* and *MyoD* gene expression domains followed by muscle cell differentiation, attested by myosin synthesis. This leads to an hypertrophy of muscles in vivo. We also show that addition of SHH in myoblast primary cultures leads to an increase in BrdU incorporation followed by an increase of myotube number.

SHH activates proliferation of muscle cells

One of the striking effects of SHH revealed in these experiments concerns the increase of myoblast proliferation. It seems unlikely that SHH converts fibroblasts into myoblasts, since neither fibroblasts transiently transfected with Shh/RCAS nor a SHH/pBK-expressing cell line express any muscle genes (data not shown). The results demonstrate that SHH activates proliferation of already committed myoblasts which results in an increase in myotube number in vitro and in hypertrophy in vivo. This effect is not mediated by FGF-4 (see Results). Although FGFs are known to induce proliferation of myoblasts, they also inhibit transcription of the myogenic bHLH proteins, leading to an inhibition of terminal differentiation (Olson, 1992).

The proliferative effect of SHH is consistent with the observation that SHH is able to stimulate BrdU incorporation in Pax-1-positive cells in the sclerotome of somite explants (Fan et al., 1995). Furthermore, it has been shown recently that SHH increases proliferation of retinal precursor cells in vitro (Jensen and Wallace, 1997) and of mesenchyme and epithelial cells during lung development (Bellusci et al., 1997). SHH could be a mitogenic factor acting like a growth factor for various cell types. Supporting this hypothesis, a mutation in the SHH receptor PTC that mimick an anarchic activation of HH signalling (Stone et al., 1996; Marigo et al., 1996a) leads to Gorlin syndrome (basal cell nevus syndrome, BCNS) (Johnson et al., 1996; Hahn et al., 1996, Oro et al., 1997). This syndrome is characterised by many developmental abnormalities and interestingly, these include myogenic tumors (Gorlin, 1990).

The premature disapperance of *MyoD* mRNA in the Shh/RCAS-infected wing at 10 days (Fig. 6) can be interpreted as an acceleration of differentiation, since *MyoD* transcripts decline to low expression levels in adult muscle tissue (Hinterberger et al., 1991). Furthermore, It has been shown recently that ectopic expression of SHH, in zebrafish, leads to the formation of ectopic slow muscle at the expense of fast muscle (Bladgen et al., 1997).

SHH is a candidate for mediating the action of the neural tube/notochord complex on axial myogenesis in chick (Johnson et al., 1994; Münsterberg et al., 1995; Maroto et al., 1997) and in zebrafish (Weinberg et al., 1996; Currie and Ingham, 1996). Targetted disruption of the *Shh* gene in the mouse leads to a reduced accumulation of *Myf-5* transcripts in myotome (Chiang et al., 1996). Consistent with the notion that SHH is the neural tube/notochord signal acting on epaxial myogenesis, the degeneration of axial muscles following

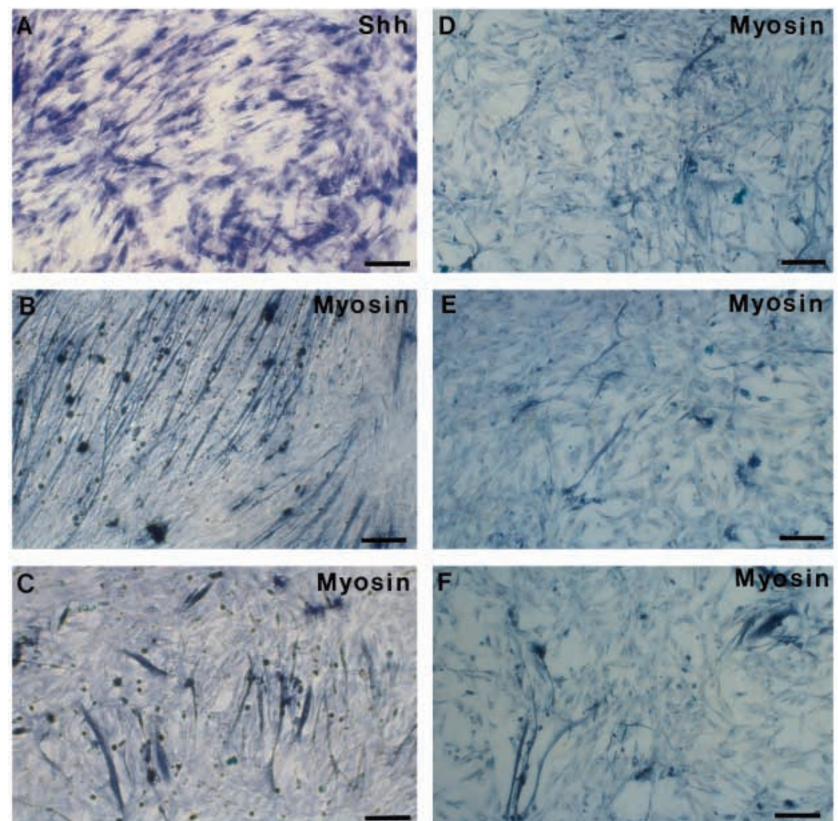

Fig. 7. Effect of SHH on primary cultures of myoblasts from 7-day-old chick embryos. Three-day primary culture of myoblasts grown in supernatant from Shh/RCAS-expressing cells (A,B), in supernatant from SHH/pBK-transfected QT6 cells (C), in medium alone (D), in supernatant from RCAS-expressing cells (E) and in supernatant from QT6 cells (F). Cultures were stained with the monoclonal antibody MF20 against sarcomeric muscle myosin, except in A where the culture was hybridised with a digoxigenin-labelled antisense RNA probe specific for chicken *Shh*. Scale bars, 50 μm.

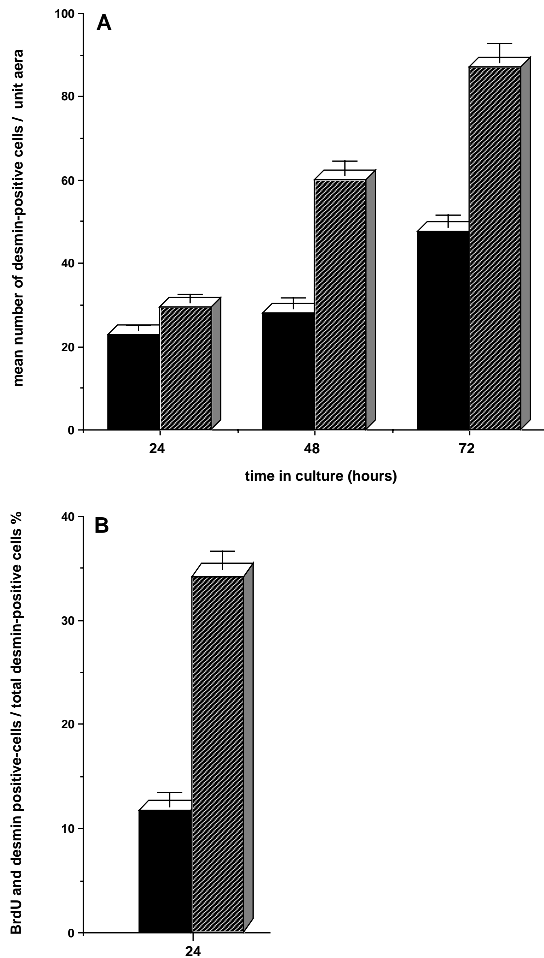

Fig. 8. Effect of SHH on myoblast proliferation in culture. Cultures were derived from pectoral muscle of 7-day chick embryos and were plated with conditioned medium from control cells or from Shh/RCAS-expressing cells. (A) The cultures were stopped 24, 48 and 72 hours later and were stained with a desmin antibody and counterstained with hemalun-eosin, in order to count nuclei of muscle cells. Each point represents the average number of nuclei per 0.135 mm^2 for 20 randomly chosen squares from four or five independent experiments. (B) Twenty-four hour myoblast culture with or without SHH-conditioned medium were pulsed with BrdU (10 μM) for the last 8 hours and processed for double immunocytochemistry using an anti-BrdU antibody and a desmin antibody. Means numbers of the desmin- and BrdU-positive cells were counted and compared to the mean total number of desmin-positive cells in each field (0.135 mm^2). Each point represents the mean over all fields of the fraction of desmin positive cells that are BrdU positive. Counts were made in 40 randomly chosen fields from two independent experiments. Vertical lines through each point represent the standard errors of the mean. Black columns, control cultures. Shaded columns, Shh/RCAS-treated cultures.

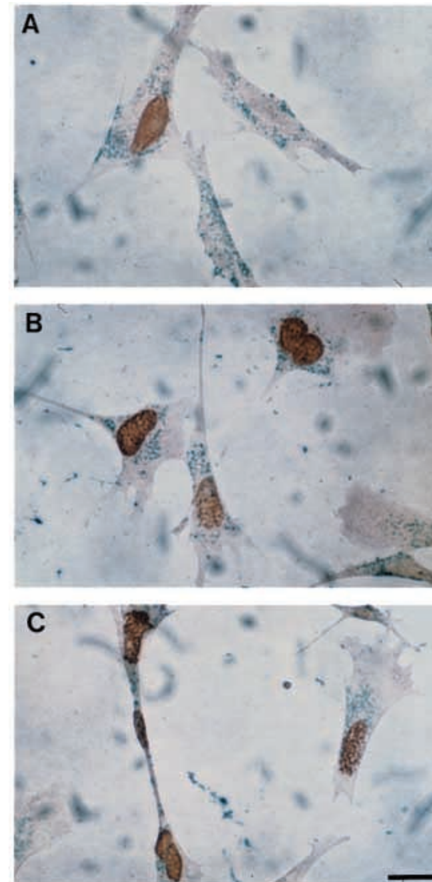

Fig. 9. SHH induces more incorporation of BrdU in desmin-positive cells. Twenty-four hour myoblast culture with or without SHH-conditioned medium, pulsed with BrdU (10 μM) for the last 8 hours, were processed for double-immunocytochemistry using an anti-BrdU antibody and a desmin antibody. Peroxidase activity (revealed by diaminobenzidine tetrahydrochloride) staining reveals BrdU-positive nuclei in brown while alkaline phosphatase staining shows the desmin labelling in blue. Desmin immunostaining in myoblasts appears in the form of dots scattered throughout the cytoplasm, reflecting the non-organised myofilaments as already described for other myofibrillar proteins. Myoblast culture with conditioned medium from control cells (A), with conditioned medium from Shh/RCAS-expressing cells (B,C). There are more BrdU-positive cells in B than in A. C is a different field from the same culture as B, showing the beginning of the fusion process between myoblasts. Scale bar, 12.5 μm.

ablation of the neural tube/notochord complex (Teillet and Le Douarin, 1983; Rong et al., 1992) can be prevented by implantation of SHH-expressing cells (Teillet et al., unpublished). This result shows that SHH acts as a survival factor on the epaxial muscle lineage (Teillet et al., unpublished). In the notochord/neural tube experiments, it was striking to see that in contrast to the epaxial muscle precursors, their hypaxial counterpart could survive and further differentiate. This suggested that a signal for the survival and growth was likely to be provided to these cells in the limb bud itself. The results presented here suggest that SHH protein produced in the ZPA may play a similar role in the limb bud, promoting the survival/proliferation of the Pax-3-positive cells. These cells then undergo the myogenic programme characterised by the activation of *MyoD* gene expression and then by the expression of muscle terminal markers such as myosin. Consistent with this hypothesis, it has been shown recently that application of SHH in combination with WNT-1 induces somitic expression of *Pax-3* prior to that of *MyoD* in presegmented explants, in vitro. Moreover, retroviral overexpression of Pax-3 activates *MyoD* and *myosin*

production in presegmented explants (Maroto et al., 1997). This suggests the existence of an hierarchy in which SHH activates Pax-3 expression which in turn activates MyoD. A link between this signalling cascade and cell proliferation may be provided by the tyrosine kinase receptor, C-met, which is thought to be under the influence of Pax-3 (Yang et al., 1996; Datson et al., 1996; Epstein et al., 1996). Hepatocyte Growth Factor (HGF), ligand of this receptor, is believed to be involved in many processes where cell growth and migration are required. Recently it has been shown that HGF/Met in addition to its effect on myoblast migration (Bladt et al., 1995; Heymann et al., 1996) could sustain proliferation/survival of feotal myoblasts from which secondary fibres are formed (Maina et al., 1996).

Proliferation of Pax-3-positive muscle precursors cells are probably under the influence of a combination of positive and negative regulatory factors. Disruption of a new TGFβ family member, growth/differentiation factor-8 (GDF-8), leads to an hypertrophy of muscle (MacPherron et al., 1997), suggesting that GDF-8 might act as a negative regulator of skeletal muscle growth.

Which signalling pathway mediates the effect of SHH on muscle?

Drosophila studies have identified several genes that appear to be indicative of HH activity (see for a review, Perimon, 1995). Among them, the segment polarity gene *smoothened* (*smo*) is required for the response of cells to hedgehog signalling during development of both the embryonic segments and the imaginal disc (Van den Heuvel and Ingham, 1996; Alcedo et al., 1996). The gene *smo* has recently been cloned in the rat (*rSmo*) and appears to be expressed in skeletal muscle (Stone et al., 1996), suggesting that smo might be required for the SHH effect on muscle.

cAMP-dependant protein kinase (PKA) appears to be a common negative regulator of HH signalling in insects (for a review, Perimon, 1995) and vertebrates (Hammerschmidt et al., 1996). Inactivation of PKA activity mimics overexpression of HH (Hammerschmidt et al., 1996; Epstein et al., 1996; Concordet el al., 1996), whereas constitutive activation of PKA activity blocks endogeneous and ectopic SHH effects (Fan et al., 1995; Hammerschmidt et al., 1996). Furthermore, it is well established that overexpression of PKA inhibits myogenic differentiation by specifically down-regulating the myogenic bHLH proteins, MYOD and MYF5 (Li et al., 1992; Winter et al., 1993). We have no evidence that the SHH effect we observe on muscle lineage in the chick limb bud is mediated by the inhibition of the PKA activity, but it seems plausible that this occurs.

Range of SHH action

The effect of SHH on muscle cells suggests that either the SHH protein diffuses beyond its expression domain or the effect is mediated by another factor. Pax-3 and MyoD-positive cells do not overlap with Shh-positive cells, but do overlap with the *Ptc* expression domain (see Results). *Ptc* expression has been already detected throughout the dorsal portion of the late neural tube, while *Shh* is restricted to the floor plate, leading to the suggestion that SHH could diffuse and act beyond its expression site (Marigo and Tabin, 1996). Furthermore, it has been shown that SHH mediates the notochord signal to the sclerotome over a distance of 150 μm (Fan et al., 1995). This effect is mediated by a direct action of the amino product of HH (Fan et al., 1995). In contrast, the induction of floor plate differentiation by SHH expressed in the notochord required short range signalling (Roelink et al., 1994, Tanabe et al., 1995). It has been recently shown that a tethered form of SHH has a full polarizing activity suggesting that SHH does not need to diffuse to have its effect on digit patterning (Yang et al., 1997). This does not exclude a long range effect of SHH on muscle cells.

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